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Measurement of messenger RNA encoding the α -chain, polymeric immunoglobulin receptor, and J-chain in duodenal mucosa from dogs with and without chronic diarrhea by use of quantitative real-time reverse transcription-polymerase chain reaction assays

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Objective—To examine the difference in expression of messenger RNA (mRNA) transcripts for polymeric immunoglobulin receptor (pIgR), α -chain, and J-chain determined by use of quantitative real-time reverse transcription-polymerase chain reaction (QRT-PCR) assays in duodenal biopsy specimens obtained from dogs with and without chronic diarrhea.

Sample Population—Biopsy specimens of the proximal portion of the duodenum were obtained endoscopically from 39 dogs evaluated because of chronic diarrhea (12 German Shepherd Dogs and 27 non-German Shepherd Dog breeds); specimens were also obtained from a control group of 7 dogs evaluated because of other gastrointestinal tract diseases and 2 dogs that were euthanized as a result of nongastrointestinal tract disease.

Procedure—Dogs were anesthetized, and multiple mucosal biopsy specimens were obtained endoscopically at the level of the caudal duodenal flexure by use of biopsy forceps; in 2 control dogs, samples were obtained from the descending duodenum within 5 minutes of euthanasia. One-step QRT-PCR was used to quantify the level of expression of transcripts for the housekeeper gene glyceraldehyde-3-phosphate dehydrogenase, pIgR, α -chain, and J-chain in duodenal mucosal tissue.

Results—There was no significant difference in the level of expression of any transcript among non-German Shepherd Dog breeds without diarrhea (control group), non-German Shepherd Dog breeds with chronic diarrhea, and German Shepherd Dogs with chronic diarrhea.

Conclusions and Clinical Relevance—Results indicated that the susceptibility of German Shepherd Dogs to chronic diarrhea is not a result of simple failure of transcription of the key genes that encode molecules involved in mucosal IgA secretion. (*Am J Vet Res* 2005;66:11–16)

Immunoglobulin A plays an important role in immune responses of the gastrointestinal tract large-

ly because it is present in mucosal secretions at concentrations far in excess of other immunoglobulin classes.¹ It prevents colonization and invasion by microorganisms, neutralizes bacterial toxins, and eliminates antigen from the lamina propria via transport of immune complexes.² Most of this IgA is locally produced by IgA⁺ plasma cells within the lamina propria³ and is transported across the epithelial barrier by the polymeric immunoglobulin receptors (pIgRs). Critical to this transport mechanism is the presence of the J-chain that links the IgA monomers to form dimeric IgA.⁴ A portion of the pIgR, termed the secretory component, remains bound to the dimeric IgA after proteolytic cleavage of the pIgR from the apical membrane of the enterocyte, forming secretory IgA.⁵ The secretory component increases the resistance of IgA to proteases present within mucosal secretions.^{6–8}

Selective IgA deficiency is the most common primary immunodeficiency in humans and affects approximately 1 in every 500 Caucasians.⁹ Human IgA deficiency has been associated with several diseases including allergies, autoimmunity, recurrent respiratory tract infections, and gastrointestinal tract disease, but most IgA-deficient humans do not have serious associated complications and are clinically normal.¹⁰ Immunoglobulin A knockout mice have a decreased ability to clear infection with *Giardia* spp, compared with wild-type mice.¹¹

Secretory component deficiency has been associated with chronic gastrointestinal tract disease and malabsorption in humans.^{12,13} These reports noted a decrease in both mucosal IgA and free secretory component in affected individuals, compared with clinically normal humans.^{12,13} There is some dispute as to whether primary pIgR deficiency has been convincingly documented in humans, as some affected individuals may have pIgR deficiency secondary to the presence of other disease conditions.^{14,15}

Compared with wild-type mice, polymeric immunoglobulin receptor knockout mice have a decreased concentration of IgA within intestinal secretions despite the presence of normal or increased numbers of IgA⁺ plasma cells in the lamina propria.^{16,17} Similarly, J-chain knockout mice also have a decreased concentration of IgA in bile,¹⁸ feces,¹⁸ and intestinal secretions¹⁹; the IgA present within these secretions

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was not polymeric and was not associated with the secretory component.²⁰ Furthermore, the J-chain-deficient IgA from J-chain knockout mice could not associate with pIgR present on Madin-Darby canine kidney cells transfected with the receptor.¹⁸

German Shepherd Dogs are susceptible to several inflammatory or immune-mediated diseases including disseminated aspergillosis,²¹ deep pyoderma,²²⁻²⁵ anal furunculosis,^{26,27} small intestinal bacterial overgrowth,²⁸⁻³² and inflammatory bowel disease.³³ Studies^{29,32,34,35} of serum IgA concentrations in German Shepherd Dogs with or without disease have revealed a decreased concentration relative to that in control populations. Decreased mucosal immunity associated with this apparent deficiency in serum IgA was implicated as a factor that predisposed dogs of this breed to small intestinal bacterial overgrowth.²⁹ In a report of another study,³⁶ the concentration of IgA in duodenal mucosal explant culture supernatants from German Shepherd Dogs with normal numbers of IgA⁺ lamina propria plasma cells was decreased, compared with non-German Shepherd Dog breeds. A defect in the transport of IgA across the epithelial barrier, or low IgA production by the plasma cells, may be responsible for this decreased mucosal IgA secretion in German Shepherd Dogs, which in turn may play a role in the apparent susceptibility of dogs of this breed to chronic diarrhea.

The purpose of the study reported here was to examine the difference in expression of messenger RNA (mRNA) transcripts for pIgR, α -chain, and J-chain determined by use of quantitative real-time reverse-transcription polymerase chain reaction (QRT-PCR) in duodenal biopsy specimens obtained from dogs with and without chronic diarrhea. Our intent was to assess the role (if any) of deficient intestinal IgA secretion in the pathogenesis of chronic enteropathies in dogs.

Materials and Methods

Dogs—Endoscopic biopsy specimens of duodenal mucosa were obtained from 39 dogs that were evaluated at the School of Clinical Veterinary Science, University of Bristol, because of chronic diarrhea. Because of limited access to clinically normal dogs, control biopsy specimens of duodenal mucosa were obtained from 7 dogs that did not have diarrhea but were undergoing endoscopy for investigation of primary gastroesophageal disease and from 2 dogs that were euthanatized with their owners' permission for clinical reasons relating to nongastrointestinal tract disease and were examined postmortem.

Dogs were separated into 3 groups on the basis of breed and clinical signs. A control group (group 1) comprised 9 dogs without chronic diarrhea for which histologic examination of contemporaneously collected biopsy specimens of duodenal mucosa revealed no abnormalities; this group included biopsy specimens obtained from 2 dogs postmortem. Breeds represented in the control group included 1 each of Rough Collie, Lurcher, Greyhound, West Highland White Terrier, Whippet, Golden Retriever, Staffordshire Bull Terrier, Flat-Coated Retriever, and crossbred. The median age of dogs in the control group was 24 months (range, 6 to 96 months), and there were 4 females (2 neutered) and 5 males (4 neutered). The diagnoses for the 7 dogs from which biopsy specimens were obtained endoscopically were chronic gastritis (n = 6) and megaesophagus (1).

Two further groups were composed of dogs with chronic diarrhea at the time of evaluation. Group 2 consisted of 27 dogs of non-German Shepherd Dog breeds; the median age of these dogs was 36 months (range, 6 to 144 months), and there were 12 females (7 neutered) and 15 males (6 neutered). Breeds represented in group 2 included crossbred (n = 5), Labrador Retriever (4), Golden Retriever (4), Boxer (2), Border Collie (2), and 1 each of Cocker Spaniel, Cavalier King Charles Spaniel, Lurcher, Old English Sheepdog, Rottweiler, Shetland Sheepdog, English Springer Spaniel, Staffordshire Bull Terrier, Tibetan Terrier, and West Highland White Terrier. Group 3 consisted of 12 German Shepherd Dogs; the median age of these dogs was 36 months (range, 12 to 132), and there were 5 females (2 neutered) and 7 males (3 neutered).

Sample collection—Dogs were prepared for endoscopy by withholding food for 18 to 24 hours. Dogs were anesthetized, and gastroduodenoscopy was performed by use of a flexible video endoscope.^a Multiple mucosal biopsy specimens were obtained at the level of the caudal duodenal flexure by use of biopsy forceps^b; collection of biopsy specimens was generally performed by 1 endoscopist (EJH). Samples for histologic examination were placed in neutral-buffered 10% formalin. Biopsy specimens for mRNA analysis were placed in a 1.0-mL cryotube,^c snap-frozen in liquid nitrogen, and stored at -70°C .

In 2 control dogs, samples were obtained from the descending duodenum within 5 minutes of euthanasia (achieved via IV administration of an overdose of pentobarbitone). Samples were obtained by use of biopsy forceps from an area equivalent to that used to provide the vital samples, snap frozen, and stored as described. Full-thickness samples were collected for histologic examination.

Primer and probe design—The primer^d and probe^e sequences for the quantification of glyceraldehyde-3-phosphate dehydrogenase (G3PDH), pIgR, and J-chain are the same as those used previously (Appendix).³⁷ A primer set was designed against canine immunoglobulin alpha heavy chain (α -chain) by use of the available GenBank sequence (accession No. L36871). Primer and probe sets were designed as described previously.³⁷ Hydrolysis probes were used in the quantification of pIgR and G3PDH; SYBR Green I was used for J-chain and α -chain quantification.

RNA isolation—All samples were coded prior to RNA extraction, and subsequent analysis was performed without personnel being aware of which dog was the source of each sample. Two biopsy specimens obtained endoscopically (total tissue mass, 8 to 18 mg) were added to a mechanical homogenization tube^f containing 800 μL of lysis buffer^g and processed for 45 seconds at 6.0 m/s to homogenize the tissues. A 175- μL aliquot of this lysate was processed through a spin-column purification system, which included an integral on-column DNase digestion step.^h Samples were processed according to the manufacturer's protocol, except that the RNA was eluted into 200 μL of nuclease-free water. A negative control of nuclease-free water was included.

QRT-PCR assay—Gene-specific QRT-PCR amplification of G3PDH, pIgR, J-chain, and α -chain was performed by use of 5 μL of total RNA in each reaction.^h Primers and probes (for G3PDH and pIgR analysis) were used at a concentration of 200nM and SYBR Green I (for J-chain and α -chain analysis) at a concentration of 1:100,000.ⁱ All reactants, with the exception of the forward primer, were mixed together as a master mix; aliquots were added to either a 24- or 96-well PCR plate^j prior to addition of 5 μL of the sample RNA to make a final volume of 25 μL . No-RT control reactions were made by substituting the RT enzyme mix with 2 units of Taq

DNA polymerase.^k Each sample was run in triplicate, in addition to the No-RT controls for G3PDH (n = 3), α -chain (1), pIgR (1) and J-chain (1). The reactions for each target were performed in the same plate. Test samples were randomly grouped prior to analysis.

The RT reaction was performed in a thermocycler^l by incubation at 50°C for 15 minutes (G3PDH, J-chain, and pIgR) or 55°C for 20 minutes (α -chain). The reactions were quenched on ice, and 200nM of the forward primer was added in a suitable volume of RT buffer to increase the reaction volume to 30 μ L because this procedure decreases primer-dimer formation associated with RT enzymes.³⁸ The plates were sealed and the protocol completed by incubation at 95°C for 5 minutes and then 45 cycles each of 95°C for 5 seconds and 60°C (62°C for J-chain) for 10 seconds, during which the fluorescence data were collected. The threshold cycle (Ct value) was calculated as the cycle when the fluorescence of the sample exceeded a threshold level corresponding to 10 SDs from the mean of the baseline fluorescence.

A melt curve was produced for the α -chain and J-chain assays by heating the samples from 75°C to 95°C in 0.4°C increments with a dwell time at each temperature of 10 seconds during which the fluorescence data were collected. The melting temperatures of the products were compared with those obtained from amplification of gel-purified α -chain or J-chain PCR products,^m which had been sequenced to check specificity. For samples with multiple products or with incorrect melting temperature, the Ct values were discarded. Negative results were confirmed by repetition of the QRT-PCR procedure.

Reaction efficiency—The assays for the quantification of pIgR, J-chain, and G3PDH have previously been shown to be > 95% efficient when tested against a 10-fold dilution series of RNA.³⁷ A 10-fold serial RNA dilution curve was produced in triplicate to calculate the QRT-PCR reaction efficiency for the α -chain assay. A master mix was prepared, and aliquots were added to the PCR plate prior to addition of the template into each reaction tube individually. A graph of threshold cycle (Ct) versus log₁₀ relative copy number of the sample from the dilution series was produced. The slope of this graph was used to determine the reaction efficiency by use of the following equation (Figure 1):

$$\text{Efficiency} = [10^{(-1/\text{slope})}] - 1.$$

Relative copy number calculation—To normalize all threshold cycle measurements for the other products, G3PDH was used as a housekeeper gene. As reported previously,³⁷ genomic contamination of the RNA samples for G3PDH occurred, despite DNase treatment. The difference in the mean Ct value for the RT and No-RT samples was calculated. The difference between these measurements was a minimum of 5 cycles, which indicated that the genomic DNA contributed < 3% of the template of the QRT-PCR reaction (eg, $1/2^5 \times 100$). Therefore, the G3PDH QRT-PCR measurements were used for normalization of the measurements for the other gene products.

The G3PDH correction value was determined by normalizing all measurements to a Ct value of 20 to provide a G3PDH correction value by use of the following equation:

$$\text{G3PDH correction value} = (20 - \text{Mean Ct value of the G3PDH QRT-PCR assay}).$$

The corrected target Ct measurement for each gene product was then calculated by adding the G3PDH correction value to the mean Ct value of the replicates for each of the gene targets.

$$\text{Corrected target Ct value} = \text{Mean Ct} + \text{G3PDH correction value}.$$

The QRT-PCR was run for a maximum of 45 cycles; therefore, a relative copy number for a sample with this value was set as 1. Samples with no measured Ct value were assigned a value of 0. All corrected Ct values were < 45. Because all the reactions were approximately 100% efficient, the relative number of gene copies in the sample was calculated by use of the following equation:

$$\text{Relative copy number} = 2^{\Delta\text{Ct}} \text{ (for a 100\% efficient reaction),}$$

where $\Delta\text{Ct} = 45 - \text{corrected target Ct value of the sample}$.

Statistical analyses—The relative copy number of each gene product was used as the basis for all comparisons. The results were assessed for normality both before and after transformation by use of the Kolmogorov-Smirnov test. Possible confounding effects of age, sex, and neutering were assessed for each gene target. The data were not normally distributed either before or after transformation; therefore, the nonparametric Kruskal-Wallis analysis of variance and the Spearman rank correlation were used as appropriate for all comparisons. Posttest analysis was performed by use of the Mann-Whitney test. The level of significance was set to $P < 0.05$ for all analyses.

Results

QRT-PCR assay optimization—A range of primer annealing temperatures was tested for the α -chain primer set in the range of 55° to 70°C by use of the gradient function on the thermocycler. The reaction was efficient at 60°C with no improvement in sensitivity at higher or lower annealing temperatures. The magnesium sulfate concentration was not increased from 3mM in the QRT-PCR assays to minimize primer-dimer formation.³⁸ The primer set used to quantify α -chain mRNA in the present study was equally able to detect the allelic variants of the canine alpha heavy chain gene³⁹; these variants differ in the hinge encoding region of the second exon, which was amplified by this primer set (data not shown).

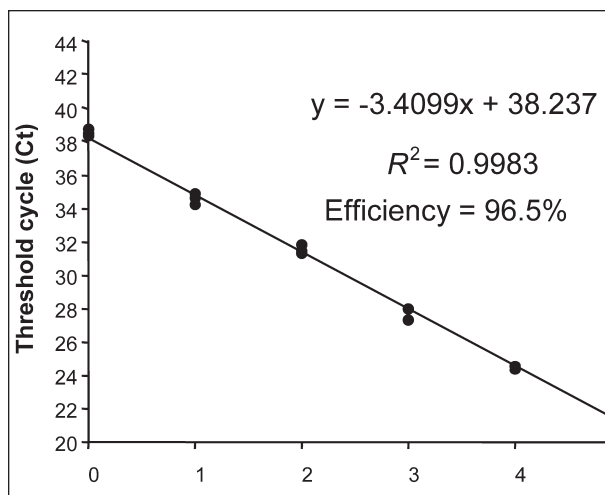


Figure 1—Standard dilution curve (threshold cycle [Ct] vs log₁₀ relative copy number of the sample) for the quantitative real-time reverse transcription-polymerase chain reaction (QRT-PCR) assay for α -chain produced from a triplicate reaction for α -chain with a 10-fold serial dilution of RNA isolated from duodenal mucosal biopsy specimens obtained from a dog. The slope of the graph was used to determine reaction efficiency by use of the following equation: $(10^{(-1/\text{slope})}) - 1$. y = The equation of the standard curve. R^2 = Correlation coefficient.

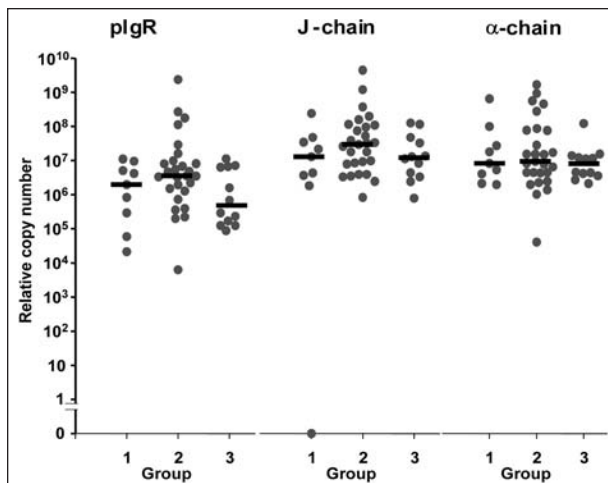


Figure 2—Plots of the relative copy number assessed via QRT-PCR assays for polymeric immunoglobulin receptor (pIgR), J-chain, and α -chain templates in duodenal biopsy specimens obtained from 9 non-German Shepherd Dogs without diarrhea (group 1), 27 non-German Shepherd Dogs with diarrhea (group 2), and 12 German Shepherd Dogs with diarrhea (group 3). The horizontal line corresponds to the median value of that group. The sample with a relative copy number of 0 had no detectable J-chain template. In the expression of pIgR, J-chain, or α -chain, there were no significant differences among the groups.

Clinical samples—The 3 specific gene targets could be quantified in all samples with the exception of J-chain, which could not be quantified in 1 RNA sample although the other 2 targets, as well as G3PDH, were detected. All copy numbers were calculated so that a corrected Ct value of 45 corresponded to a relative copy number of 1; this allowed the relative abundances of the templates to be compared. The least abundant template was pIgR, and there was approximately 10-fold more J-chain and α -chain in the RNA samples. None of the No-RT control samples for pIgR, α -chain, or J-chain produced a detectable product.

There was no correlation with age, sex, or histopathologic finding for any of the gene targets. There was no significant difference in the expression of pIgR, J-chain, or α -chain ($P = 0.199, 0.330$, and 0.712 , respectively, with the Kruskal-Wallis test) between the 3 groups of dogs (Figure 2).

Discussion

There was variation among the individual dogs in the expression levels of mRNA encoding pIgR, J-chain, or α -chain, but the ranges of values were similar among the 3 groups. Furthermore, there was no difference in the level of expression of mRNA for these 3 target transcripts between dogs with and without diarrhea or between dogs of different breeds. However, RNA can undergo posttranscriptional regulation; within a cell, RNA can be translationally active or inactive and can vary in its stability. Therefore, it is well recognized that the level of expression for a particular protein may not necessarily reflect the amount of mRNA encoding it.^{40,41} Yet results of in vitro studies have indicated that the protein expressions of human pIgR⁴² and α -chain⁴³ do correlate with their mRNA expressions. Therefore, the lack of a difference in the level of expression of mRNA

for the 3 target transcripts among dogs of different breeds and among dogs with and without diarrhea probably reflects a similar lack of difference in the expression of these proteins.

The wide variation in expression of the transcripts among dogs may be a consequence of the method of sample collection because biopsy specimens obtained endoscopically are usually superficial and generally do not include a standard ratio of crypt to villous tissue. The number of IgA⁺ plasma cells within the duodenal mucosa of dogs is greater in the pericrypt regions, compared with the number in the villus lamina propria⁴⁴; therefore, biopsy specimens with greater amounts of crypt material will have larger numbers of this type of cell. The depth of the biopsy specimen could also affect the amount of pIgR mRNA because, in humans, there is greater expression of this molecule by the epithelial cells of the crypt region.¹⁵ In the present study, this variation in the nature of the tissue sample was minimized by collecting samples by use of 1 type of biopsy forceps from a consistently designated area of the duodenum. Moreover, collection of biopsy specimens was generally performed by 1 endoscopist. Grossly undersized or poor-quality biopsy specimens were discarded. The variation in J-chain mRNA expression may reflect the relatively larger number of cell types in which this molecule is expressed, including IgA, IgM, and IgG plasma cells.^{45–48}

Our study was restricted to the investigation of duodenal mucosa because the samples were obtained during endoscopic examination of the upper portion of the gastrointestinal tract as part of clinical investigation of client-owned animals. The greatest number of IgA⁺ plasma cells is present in the lamina propria of the duodenum and colon in dogs, with fewer cells in the more distal regions of the small intestine.^{44,49,50} Peyer's patches contain populations of plasma cells, including IgA⁺ plasma cells,⁵¹ and could have been used to examine α -chain and J-chain expression; however, M cells do not express pIgR and therefore do not participate in pIgR-mediated transport of IgA.⁵² Duodenal mucosa was also selected, as previous studies in which a relative deficiency in gastrointestinal mucosal IgA secretion in German Shepherd Dogs was detected used duodenal luminal fluid²⁹ and duodenal explant cultures.³⁶

Compared with similar cells from clinically normal humans, peripheral blood mononuclear cells from IgA-deficient humans produce minimal amounts of α -chain mRNA when stimulated in vitro by anti-CD40 antibody.⁵³ In the present study, there was no evidence of decreased α -chain mRNA production in German Shepherd Dogs with chronic diarrhea, compared with production in non-German Shepherd Dogs with or without chronic diarrhea, which does not support the presence of this type of simple genetic abnormality in dogs of this breed.

Our data have indicated that mRNA transcripts for pIgR, α -chain, and J-chain in biopsy specimens of duodenal mucosa obtained endoscopically from dogs can be quantified by QRT-PCR procedures. However, no evidence was found of a simple deficiency of any of these transcripts in the intestinal mucosa of German Shepherd Dogs with diarrhea, compared with dogs of

other breeds with or without diarrhea. These observations do not rule out the possibility of a deficiency in the amount of synthesized protein or a mutated nucleotide sequence affecting the function of the encoded protein, but there is no evidence that lack of gene transcription plays a role in the disease susceptibility of this breed.

- a. GIF-XQ230, Olympus Keymed, Southend-on-Sea, UK.
- b. FB-25K, Olympus Keymed, Southend-on-Sea, UK.
- c. NUNC, Fischer Scientific Ltd, Loughborough, Leicestershire, UK.
- d. Invitrogen Ltd, Paisley, Scotland.
- e. Cruachem Ltd, Glasgow, Scotland.
- f. Green Ribolyser Tube, Ribolyser system, Thermo-Hybaid, Ashford, Middlesex, UK.
- g. SV total RNA isolation system, Promega Corp, Madison, Wis.
- h. Platinum quantitative RT-PCR thermoscript one-step system, Invitrogen Ltd, Paisley, Scotland.
- i. Sigma-Aldrich Ltd, Poole, Dorset, UK.
- j. Thermofast, Abgene, Epsom, Surrey, UK.
- k. Platinum Taq DNA polymerase, Invitrogen Ltd, Paisley, Scotland.
- l. iCycler iQ, Bio-Rad Laboratories Ltd, Hercules, Calif.
- m. QIAquick PCR purification kit, Qiagen Ltd, Crawley, UK.

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Appendix

Combinations of forward and reverse primers and probe sequences used to measure mRNA encoding glyceraldehyde-3-phosphate dehydrogenase (G3PDH), polymeric immunoglobulin receptor (pIgR), the J-chain, and the α -chain, in duodenal mucosa from dogs with and without chronic diarrhea by use of quantitative real-time reverse transcription-polymerase chain reaction assays.

Target	Forward primer*	Reverse primer*	5' Fluorophore	Probe Sequence†	3' Quencher	Product length (bp)
G3PDH	TCAACGGATTGGCCGTATTGG	TGAAGGGGTCATTGATGGCG	Hex	CAGGGCTGCTTTTAAGTCTGGCAAAGTGA	BHQ-1	90
pIgR	ATCACCAACTTCCCAGACAG	TAATGCCAGACCACACTTAT	CY5	GTGATGAACATTGACCACCTCACCCAG	BHQ-2	94
J-Chain	TCTGATCCCACTCACCAGT	TGCGAGGCAATAACTACTTGA	SYBR Green I			113
α -chain	CGTCTGTGAAATGCCAAGTG	AGGGCTGGCTTCTGTAGTGA	SYBR Green I			136

*All primers were desalted when purified. †The probes were purified via high-pressure liquid chromatography.